CHEMICAL INVESTIGATIONS OF THE TOBACCO PLANT

X. Determination of Organic Acids by Ion Exchange Chromatography

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INTRODUCTION

In recent years, the importance of aliphatic organic acids in plant and animal metabolism has become widely recognized. This laboratory has been especially concerned since 1930 with the role of organic acids in the metabolism of the tobacco plant, and analytical methods for the determination of malic, citric, oxalic, and succinic acids, and of the total organic acidity were developed (9, 14, 15, 16) for use in this work. Although the precision and accuracy of these methods are satisfactory, they are time-consuming and require careful attention to detail; improvements were urgently needed. Furthermore, it was of the utmost importance to develop methods whereby hitherto unknown acids could be detected, identified, and eventually determined quantitatively even though present in only small proportions.

The most promising of the various methods described in the literature appeared to be those based upon the chromatographic separation of individual organic acids. Of these, two types are commonly used, partition chromatography on silica gel and elution chromatography on anion exchange resins. The former depends upon the distribution of the organic acids between water held by the silica gel as an immobile phase and an immiscible organic solvent which percolates down through the column. It is thus, in effect, a continuous liquid-liquid extraction procedure. The latter depends upon the fact that organic acid anions combine with positively charged groups on a synthetic organic resin of suitable composition and remain fixed to it until displaced by an aqueous solution of an acid which percolates through the column.

Partition chromatography on silica gel or similar media, although a widely used and effective means for separating organic acids from each other (10), has serious disadvantages for routine analysis or in isolation work. Among these disadvantages are the low capacity of the partition system and the difficulty of preparing suitable samples in a volume sufficiently small so that addition to the column does not upset the sensitive equilibrium relationships which must be established and maintained.

A number of workers have employed anion exchange resins for the separation of organic acids. For example, Schenker and Rieman (17) have separated malic, tartaric, and citric acids present in extracts of fruits, and Owens, Goodban, and Stark (13) have studied the organic acids in sugar beet liquors. However, the technique of Busch, Hurlbert, and Potter (4) appeared to be especially well adapted for quantitative determination of the various acids and, in addition, offered a means for the detection and eventual determination of unknown components. These authors developed their technique specifically for the separation of the organic acids of the tricarboxylic acid cycle in animal tissues and applied it to the determination and purification of acid components labeled with radioactive carbon.

This bulletin describes modifications and extensions of the ion exchange chromatographic technique of Busch, Hurlbert, and Potter (4) which are designed to satisfy the requirements of rapidity, accuracy, and precision essential for a method to be used for routine analytical work. The modified technique is especially adapted for the rapid determination of malic, citric, and total organic acids in tobacco leaf tissue. Data are also presented on the ion exchange separation of a number of other acids of metabolic interest, and the general applicability of the technique to the analysis of other plant tissues and to certain food products is outlined.

MATERIALS

Ion Exchange Resin

Dowex[1] 1-X10 of 200 to 400 mesh size was used except where noted. The finer particles were removed by suspending the resin in water, allowing the coarser particles to settle for 20 to 30 minutes, and decanting the cloudy supernatant fluid. This operation was repeated several times. The resin was then converted to the formate or acetate form by passing a 1 *N* solution of sodium formate or sodium acetate through a column 3 cm. in diameter and 15 cm. in height containing about 100 ml. resin bed volume. This yields sufficient resin for from 40 to 50 analytical columns. The salt solution was passed through the column at a rate of from 1 to 2 ml. per minute until the effluent gave a negative or very slight test for chloride. The resin was then washed with several bed volumes of water and was stored as a fairly thick suspension in a brown bottle.

Organic Acids

The organic acids used were commercial products of the highest purity obtainable, with the exception of the isocitric acid and isocitric lactone which were prepared from dried Bryophyllum leaf by Dr. D. G. Wilson of this laboratory.

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[1]The author wishes to thank the Dow Chemical Co., Midland, Michigan, for liberal samples of Dowex 1 in the chloride form used in the development of the present technique. The designation Dowex 1-X10 indicates a styrene-divinylbenzene resin with strongly basic properties prepared so as to have approximately 10 per cent cross-linkage.

GENERAL PROCEDURE

Preparation of Tobacco Leaf Extracts

Ether Extract

Freshly picked tobacco leaves are dried at 80° C. for 2 to 3 hours in a ventilated oven with fan-driven circulation of air, broken up and equilibrated to constant moisture content by storage in open wide-mouth bottles at 24° C. and 50 per cent relative humidity for several weeks. The tissue is then ground in a Wiley mill and kept in tightly closed bottles in the air-conditioned room. Cured or cured and fermented tobacco is prepared in exactly the same way.

The extraction apparatus[1] consists of a small coil of block tin tubing attached to a plate that serves as a cover for a special 400 ml. wide-mouth Erlenmeyer flask. A glass siphon cup is suspended from the coil by means of a stirrup of copper wire.

To prepare the extract, exactly 0.500 gm. of dried tissue is thoroughly mixed with 1.0 ml. of 4 *N* sulfuric acid in a small beaker; 1.0 gm. of fine, ignited asbestos fiber is added, and the whole mixed until a homogeneous mass is obtained. Angular quartz pebbles (about 5 mm. size) are added to the glass siphon cup of the extraction apparatus to a depth of about 1.5 cm. and covered with a folded pad of several thicknesses of ether-extracted cheesecloth. The sample is then evenly packed into the cup. A second cheesecloth pad is used to wipe the last traces of tissue from the beaker and glass rod, and is gently pressed down above the sample so as to cover it completely. The sample should occupy the middle third of the depth of the glass cup, and sufficient cheesecloth (i.e., several layers) should be used to prevent the loss of any particles of tissue during extraction.

The siphon cup is suspended from the condenser coil of the extraction apparatus, and extraction with approximately 150 ml. of specially purified ether[2] is conducted for about 16 hours (usually overnight) at a siphoning rate of 40 to 60 cycles per hour.

Several drops of phenol red indicator solution and 5 ml. of water are then added to the ether in the extraction flask, and 1 *N* NaOH is added drop by drop with shaking until the indicator in the water phase becomes a deep red color. The mixture is allowed to stand for about 15 minutes with occasional shaking to make certain that all of the organic acids have been neutralized and transferred to the water phase as shown by persistence of the red color. If the color fades, further addition of alkali is made as necessary. The ether is then evaporated by a stream of compressed air. The aqueous phase is transferred to a 25 ml. volumetric flask and made to volume. The solution is filtered through a dry filter paper to remove any insoluble material. Extracts so prepared range in color from a deep, almost opaque, green to a dark brown. The pH should lie between the limits 6.5 to 10.5. The upper limit

[1]Fisher Scientific Co. catalogue No. 9-585.

[2]Ether for this purpose is prepared by washing reagent ether three times with water containing a small quantity of potassium permanganate followed by distillation over potassium hydroxide pellets.

should not be exceeded since the presence of a gross excess of alkali affects the behavior on the chromatographic column.

Water Extract

A 0.500 gm. sample of the dried and finely powdered tobacco leaf tissue is weighed into a 25 ml. centrifuge tube, and 10 ml. of water are added slowly down the side. The tube is heated in a steam bath for 15 minutes, the contents being occasionally stirred with a glass rod. The rod is then rinsed off, the tube is centrifuged, and the supernatant liquid is filtered through a small conical paper filter into a 25 ml. volumetric flask. The residue is washed three times with 5 ml. portions of water, being heated and stirred each time for a few minutes, and centrifuged. The fluid is filtered into the volumetric flask, and the contents are made to volume. Such extracts are slightly cloudy and of a light to dark yellow-brown color. The pH ranges from 5.3 to 5.6.

The Gradient Elution Apparatus

Gradient elution chromatography was first described by Alm, Williams, and Tiselius (1). They showed that the successive zones or bands into which the components of the mixture become separated could be narrowed considerably, and the elution peaks sharpened, by passing a gradually increasing concentration of eluting agent through the column. Choice of a proper gradient enabled them to separate the components present in several series of closely related compounds.

Experimentally, the gradient is produced by the introduction of a relatively concentrated solution of an eluting reagent which flows at a constant rate into a mixing chamber initially filled with solvent. The resulting mixture flows simultaneously into the column at the same rate. The expression (1)

$$C = C_0 \ (1 - e^{-K})$$

defines the gradient of concentration produced under these conditions where C_0 and C are, respectively, the concentrations of the solutions entering and leaving the mixing chamber, and K is the ratio of the volume of eluate collected at any time to the fixed volume of the mixing chamber.

Although the major organic acids of the tobacco leaf can be separated by elution from anion exchange columns with the use of a succession of definite but increasing concentrations of formic acid, the gradient elution technique was found to be superior in many ways. The peaks on the elution curve were sharper and more symmetrical, and the distance between peaks was greater. Furthermore, the gradient elution system runs with minimal attention, less time is required for the elution, and interruption of the process to change the eluent is unnecessary. In addition to facilitating the analytical technique, these factors allow preliminary separations of unknown complex mixtures to be made with more certainty.

The gradient elution apparatus used in this laboratory is modified from that of Busch, Hurlbert, and Potter (4) and consists of a reservoir

containing the eluting agent (formic or acetic acid) at a suitably high concentration and a mixing vessel initially containing 210 ml. of water. A 500 ml. filter flask serves as the reservoir and a 250 ml. filter flask containing a magnetically driven stirrer as the mixing vessel. The necessary connections and the arrangement for the supply of compressed air are shown in Figure 1. The mixing vessel is fitted with a capillary tubing outlet which connects through a ground glass joint to the resin column.

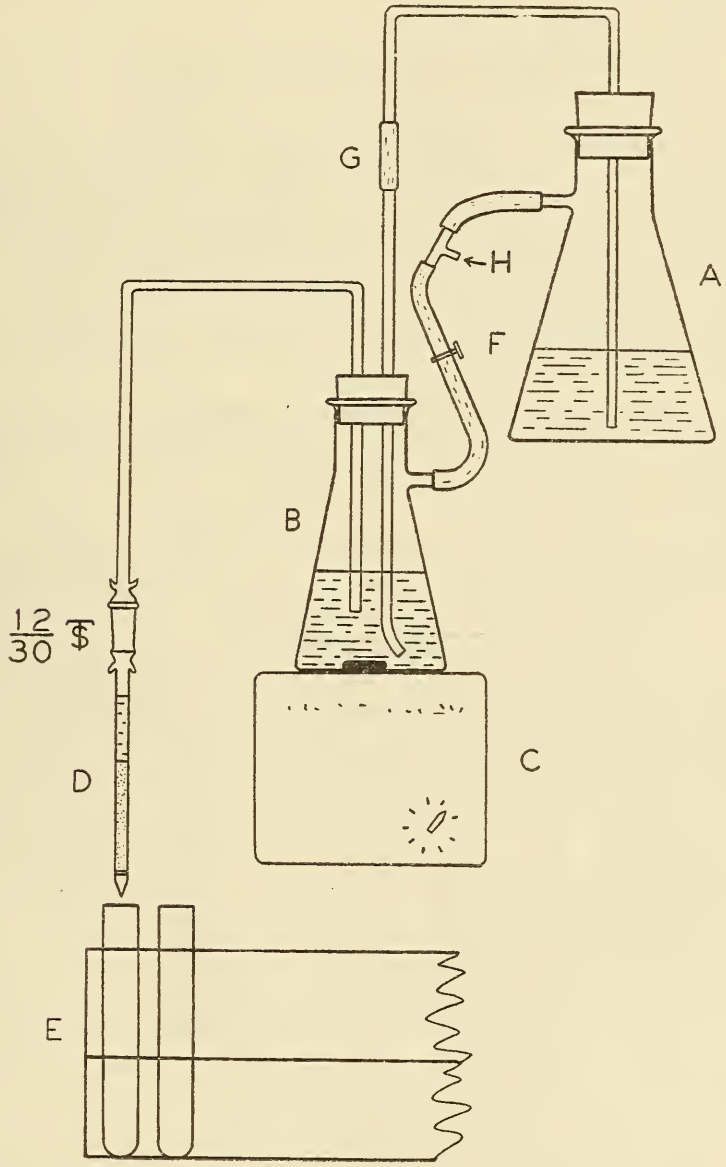

FIGURE 1

Gradient elution apparatus. A - reservoir, B - mixing vessel, C - magnetic stirrer, D - glass tube containing resin column, E - portion of fraction collector rack, F - adjustable hose clamp, G - section of rubber tubing to provide flexibility, H - inlet for air pressure. The glass tubing connecting the vessels to each other and to the resin column is heavy-walled capillary tubing of approximately 1 mm. inside diameter.

To operate the apparatus, the air pressure (about 2 pounds per square inch) is turned on with screw clamp F (Figure 1) open. Water

from the mixing vessel is thereby forced into the column; the pressure is adjusted to provide a rate of flow of effluent of about 1 ml. per minute. As soon as this rate has been established, clamp F is closed. Usually about 10 ml. of water (3 or 4 fractions) have been collected by the time the adjustment is satisfactory, and about 200 ml. of water remain in the mixing vessel. After clamp F is closed, acid begins to pass slowly over into the mixing vessel, and elution of the organic acids on the column begins. This technique avoids a sudden influx of acid into the mixing vessel.

Preparation of the Resin Column

The resin column is supported in glass tubing of 0.6 cm. inside diameter (0.28 sq. cm.) and approximately 20 cm. long constricted at the lower end so as to hold a removable alundum disc. The top of the glass tube is fitted with a ground glass joint for connection to the capillary tube coming from the mixing flask. Two circular filter paper discs, slightly larger in diameter than the tubing, are pressed down on the alundum disc so as to prevent leakage of resin.

In certain separations, in order to provide for greater capacity, it is necessary to employ a larger column prepared in a tube 1 cm. in diameter (0.78 sq. cm.) and 30 cm. long.

To prepare the resin column, the bottle containing the resin previously converted to the desired form is thoroughly shaken in order to produce a uniform, fairly thick suspension, and a portion of this suspension is poured into the glass tube. Air presure at 2 pounds per square inch is applied to the top of the tube to force the resin into place. When the layer of settled resin reaches the height desired, the air pressure is released and the excess suspension of resin is decanted. Particles that adhere to the sides of the tube are carefully washed down to the surface of the resin bed. A small piece of glass wool is then placed on top of the resin, and 1 to 2 bed volumes of wash water are passed through with the aid of air pressure. The aqueous solution of the sample is finally added and forced into the resin under 1 to 2 pounds pressure, followed by an additional 2 bed volumes of wash water. Best results are obtained if the liquid level is not allowed to fall below the top of the resin bed at any time. Approximately 1 to 2 ml. of water are added above the resin, and the column is connected to the mixing vessel. Fractions of 2.0, 2.5, or 3.0 ml. (depending on the separation desired) are collected on the fraction collector[1] at a rate of about 1 ml. per minute. The pressure of from 2 to 5 pounds per square inch required to produce this rate is maintained by a manostat[2] connected to the air line.

Since formic and acetic acids were used at several initial concentrations as the eluting acids, the exact conditions used for particular separations will be specified later.

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[1]Technicon Chromatography Corporation, New York.

[2]Moore Products Company, Philadelphia.

Analysis of the Effluent

Most of the acids were determined by titration of the free acid after evaporation of the eluting agent. Volatilization of formic acid in heated desiccators at 25 to 30 mm. of mercury as described by Busch, Hurlbert, and Potter (4) produced erratic results in our hands, and was unsatisfactory because of the time required. Advantage was accordingly taken of the fact that the heated fractions could be rapidly evaporated in a stream of air.

The 18 x 150 mm. test tubes containing the fractions are placed in order in an aluminum test tube rack[1] in a rectangular water bath[2] at 46 to 48°, and a glass manifold, constructed as in Figure 2, is placed on the row of tubes as shown. The manifold tubes are of such a length that the accurately tooled capillary openings are approximately 2 cm. above the surface of the liquid. The test tubes are submerged in the warm water for about one-third of their length. Adjustment of the air flow to a point such that the surface of the liquid is considerably agitated but no splashing occurs brings about satisfactory volatilization rates. For 3 ml. fractions from a formic acid column, the air stream is maintained for 80 minutes. Acetic acid is more readily volatilized, and 70 minutes has proved sufficient for complete removal. Evaporation of visible fluid is usually complete in 50 to 60 minutes, but slight variations in evaporation rate from tube to tube make it safer to allow the time prescribed. There is danger of loss of certain acids (especially malic acid) if the air stream is allowed to flow for an excessive time after all liquid has disappeared.

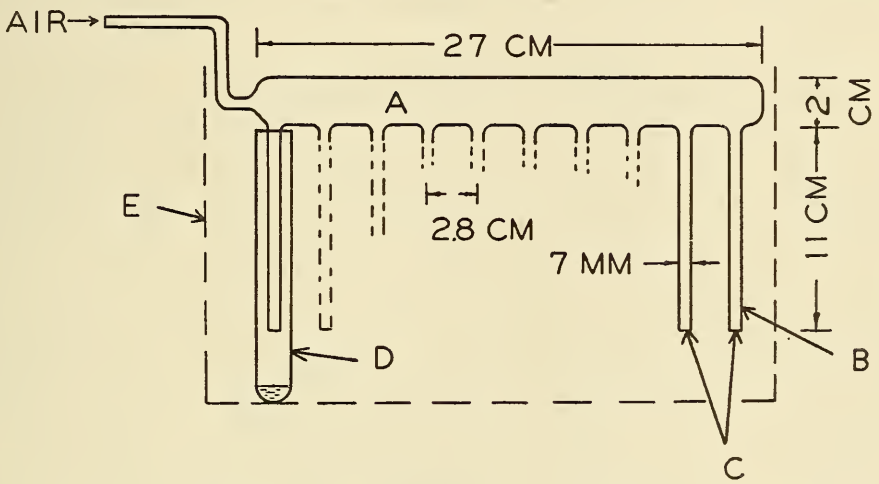

FIGURE 2

Air manifold. A - header of manifold, B - one of 10 outlet tubes of manifold, C - openings of outlet tube precisely tooled to 1 mm. diameter, D - test tube in place during evaporation, E - outline of test tube rack. The manifold outlet tubes are spaced to fit into the row of test tubes which in turn are held in the metal test tube rack. The header of the manifold rests on the lips of the test tubes and fits loosely between the raised ends of the rack.

[1]Technicon Chromatography Corporation, New York, catalogue No. 150.
[2]Labline, Inc., Chicago, catalogue No. 3012.

At the end of the drying period, the rack is removed from the water bath, and 2 ml. of CO_2-free water are added to each tube. The solutions are then titrated to the phenol red end point with CO_2-free 0.020 *N* NaOH delivered from a microburette.[1]

A separate study of the volatilization technique showed that 98 to 102 per cent recoveries of pure succinic, malic, tartaric, maleic, and citric acids could be obtained under the conditions described.[2] Examination of the column effluents indicated that equally good recoveries can be obtained for fumaric, aspartic, and glutamic acids. Malonic acid, however, consistently gave recoveries of from 102 to as much as 120 per cent when formic acid solutions of this acid were evaporated and titrated.

Glycolic, glyoxylic, glyceric, pyruvic, oxalic, and α-ketoglutaric acids were partially or wholly lost either by oxidation or volatilization or both. These acids are accordingly determined in pooled fractions from the collector, α-ketoglutaric, glyoxylic, and pyruvic acids by suitable modification of the method of Friedemann and Haugen (8) and glycolic acid by the method of Calkins (5). For these measurements, controls are usually necessary to correct for the presence of impurities in the formic acid which affect the colorimetric procedures. The determination of oxalic acid is described on page 11.

The fractions from tobacco leaf tissue that contain citric acid were found to be contaminated with a significant proportion of an as yet unidentified acid component. This necessitates the determination of citric acid by the chemical method (9) in the pooled fractions after they have been evaporated and titrated.

Rapid Procedure for Total Nonvolatile Organic Acidity

For some purposes, a measurement of the total nonvolatile organic acidity present in a sample may be the only information required. This determination can be carried out quickly without gradient elution, and a fraction collector is not required. An aliquot of tissue extract containing approximately 0.1 m.eq. of total acidity is placed on a 4.0 cm. x 0.28 sq. cm. column of formate resin with use of the procedure already described. Elution is accomplished by passing 16 ml. of 6 *N* formic acid through the column at a rate of about 1 ml. per minute. Fractions of approximately 2 ml. in volume are collected, dried, and titrated. Under these conditions, the organic acids are completely eluted in the first four fractions. Phosphoric acid, if present, can usually first be detected in fraction 6 and, accordingly, a portion of each of the later fractions may be tested after titration for the presence of phosphate using the sensitive phosphomolybdate-benzidine test described by Feigl (7). How-

[1]Kimble Glass Company, Toledo, Ohio, catalogue No. 17105.

[2]The conditions of temperature and air flow rate described were found to be optimal. Higher air flow rates brought about losses from splashing. Lower air flow rates, as well as lower temperatures (30-35°), increased the time required two- to threefold and accentuated the differences in drying time from tube to tube. At higher temperatures (60°), the time required was reduced about one-third, but 5 to 8 per cent losses of malic acid occurred.

ever, since 1 or 2 fractions having low (0.4 microequivalent or less) titrations usually occur between the fractions containing the last of the organic acidity and those in which phosphoric acid first appears, inspection of the titration values in most cases readily indicates the point at which the organic acids have been completely eluted.

As is evident from determinations on extracts of dried tobacco leaf, the sum of the acidity eluted before phosphoric acid appears represents the total nonvolatile organic acidity, exclusive of that due to oxalic acid. This substance remains on the column but ordinarily is not present in water extracts of tobacco leaf tissue in significant amounts.

Since the anions such as NO_3^-,$SO_4^=$,Cl^-, etc., which often interfere in paper chromatography, are held on the column during elution with formic acid, and neutral and basic substances (i.e., sugars, amino acids, etc., if present) are washed off the column by the preliminary treatment of the resin with water, the effluent obtained by this technique is well adapted for examination by paper chromatography. For this purpose the effluent must usually be concentrated to a small volume before testing an aliquot on paper.

Determination of Oxalic Acid

Oxalic acid is a major constituent of the organic acid fraction of the mature tobacco leaf. Determination of this acid by the ion exchange technique as at present developed presents several difficulties. A stronger acid than malic or citric acids, it requires strenuous conditions for its elution from the ion exchange column and, since free oxalic acid is somewhat volatile, it cannot be directly titrated after evaporation of the eluting agent. Direct permanganate titration of the eluted acid is impossible in the presence of formic acid. Furthermore, oxalic acid occurs in dried tobacco leaf tissue as the calcium salt, and water extracts contain less than 5 per cent of the total quantity present. However, ether extracts are suitable because oxalic acid can be quantitatively extracted from the acidified tissue with this solvent.

Accordingly, oxalic acid is most readily and accurately determined independently of the ion exchange procedure. When using ether extracts, a modification of the method of Pucher, Wakeman, and Vickery (16) is readily applied. If water extracts have been used for the extraction of the other organic acids, a modification of the method of Baker (2) may be employed to determine oxalic acid on a separate sample of the original tissue or on the residue remaining after water extraction. The final steps in both methods are separation of oxalic acid as calcium oxalate, followed by solution of the oxalate in sulfuric acid and titration with potassium permanganate. These procedures are the same regardless of which method of extraction is used.

If ether extracts have been prepared as previously described, a 5 ml. aliquot is acidified with 0.5 ml. of 2 *N* hydrochloric acid, and 50 to 100 mg. of asbestos are added. The solution is filtered with gentle suction through a small Gooch crucible which contains a thin asbestos mat into a 25 ml. centrifuge tube. The asbestos is washed with water

added in small portions to the crucible until the total volume of filtrate reaches 20 ml., and the calcium oxalate precipitation is carried out on this solution.

If water extracts have been employed for the extraction of the other organic acids, the preparation of an additional extract is desirable and, for this purpose, 0.5 gm. of the original dried tissue is required. If the supply of sample is limited, the residue after the hot water extraction of tobacco leaf tissue may be employed although slight losses of oxalic acid may then occur. In either case, the extraction procedure is the same. To a 0.5 gm. sample of the dried tissue (or its equivalent as residue) are added 10 ml. of 1 *N* hydrochloric acid; the mixture is heated for 15 minutes on the steam bath with occasional stirring. After being cooled, the suspension is transferred to a 25 ml. volumetric flask and made to volume. The mixture is shaken and allowed to stand overnight at room temperature. Insoluble material is removed by filtration through a dry paper whereby a clear, yellow extract is obtained. A 5 ml. aliquot is pipetted into a 15 ml. tapered centrifuge tube, 5 ml. of phosphoric acid-tungstate reagent[1] are added, the solution is thoroughly mixed and allowed to stand for approximately 5 hours. The precipitate is then sedimented by centrifugation at approximately 1500 *g.* for 10 minutes. A 5 ml. aliquot of the supernatant fluid is transferred to a 15 ml. tapered centrifuge tube for the calcium oxalate precipitation.

In order to precipitate the calcium oxalate, 6 *N* ammonium hydroxide is added drop by drop to the solution until it is alkaline to bromocresol purple or, in the case of water extracts, as indicated by precipitation of a small quantity of phosphotungstate; 3 ml. of calcium chloride-acetate buffer solution[2] are then added. After being thoroughly mixed with a fine glass rod which is washed and removed, the solution is allowed to stand overnight. The white precipitate is centrifuged at about 1500 *g.* for 10 minutes, and the supernatant solution carefully decanted. The sediment is washed once with 5 ml. of a 5 per cent acetic acid solution saturated with calcium oxalate, being thoroughly stirred with a fine glass rod, and is again centrifuged. The calcium oxalate is dissolved in 5 ml. of 4 *N* sulfuric acid, the solution is heated in a boiling water bath and titrated hot with 0.02 *N* potassium permanganate to a faint pink end-point which remains for 10 to 15 seconds. One ml. of 0.02 *N* permanganate is equivalent to 0.09 mg. or 0.02 m.eq. of oxalic acid.

Paper Chromatography

The technique of chromatography on paper is extremely useful for examining the efficiency of the separation on the ion exchange columns and also for preliminary studies of the unknown acids which appear as peaks on the elution curves. For such control work, a rapid and reliable system was desired. A modification of the procedure described by Denison and Phares (6) proved most satisfactory.

[1]24 gm. of sodium tungstate are dissolved in water, 40 ml. of syrupy phosphoric acid (sp. gr. 1.75) are added, and the solution is diluted to 1 liter (2).

[2]25 gm. of anhydrous calcium chloride are dissolved in 500 ml. of 50 per cent (v/v) acetic acid, and a solution of 330 gm. of sodium acetate trihydrate in sufficient water to make 500 ml. is added. The pH of the final solution is approximately 4.5 (2).

The developing solvent is a mixture of ethyl ether, 88 per cent formic acid, and water in the proportions 5:2:1. Formic acid is preferred as the reagent to repress the ionization of the organic acids since it produces more sharply defined spots than acetic acid, especially if the acids are applied to the paper as sodium salts.

The chromatograms are run by an ascending technique in 1 gallon wide-mouth screw-capped reagent bottles containing a 1 cm. depth of solvent. The inside of the cap is coated with a thin layer of paraffin. A roll of filter paper reaching almost to the top of the bottle is supported centrally by a small vial of the solvent mixture. This arrangement aids in maintaining an atmosphere equilibrated with solvent within the bottle.

To prepare the chromatogram, spots 1 cm. or less in diameter containing 0.0005 to 0.001 m.eq. of organic acid each are applied along a pencil line drawn 2 cm. from the edge of 23 x 23 cm. sheets of Whatman 3 MM paper, the spots being 2 to 3 cm. apart. Either water or acetone is used as solvent, and delivery is made from a 0.1 or 0.2 ml. pipette in small portions until the desired quantity has been applied. The pipettes are prepared for this purpose by drawing the tip out to a fine capillary which is marked with a file and broken off, the rough edges being smoothed on a fine carborundum stone. In applying the spots, the tip of the pipette is touched to the paper and the size of the moistened area is controlled with the finger. A stream of warm air from an electric hair dryer blowing vertically upwards from below the paper aids in quick drying of the spots.

The sheet is stapled into the form of a cylinder so that the edges do not quite meet, and the cylinder is placed in the bottle with the row of spots at the bottom. The lid of the bottle is then screwed down firmly. The bottles and solvent mixture are kept at a constant temperature of 25 to 26° C. At 25° C., the solvent front travels 17 to 20 cm. in 2 to 2.5 hours. At the end of this period, the paper cylinder is suspended in a length of stove pipe, and steam is introduced at the bottom for 15 minutes to evaporate the formic acid. The paper is then opened flat, dried before a fan at room temperature, and is finally sprayed with 0.04

Table 1. R_F Values of Some Common Organic Acids

Acid or salt	R_F x 100	Acid or salt	R_F x 100
Ascorbic	32	α-ketoglutaric	69
Trans-aconitic	78	Lactic	80
Aspartic	29	Lactate (zinc)	81
Citric	46	Maleic	78
Glucuronate (sodium)	13	Malic	55
Glutamic	33	Malate (sodium)	54
Glyceric	49	Malonic	74
Glycerate (calcium)	48	Mandelic	92
Glycolate (potassium)	64	Oxalic	18
Hydroxypyruvate (potassium)	29	Pyruvate (potassium)	83
Isocitric	46	Succinic	79
Isocitric lactone	61	Tartaric	33
Itaconic	82		

per cent bromophenol blue in alcohol.[1] Yellow spots on a blue background develop. The solvent front is marked by a narrow yellow line.

The R_F values of a series of acids and their salts as obtained with this technique are summarized in Table 1. Variation in the R_F values between runs was of the order of 2 to 3 units or less. Malic acid was used on each chromatogram as a control in order to reveal any gross irregularity in the R_F values. A series of 20 routine determinations of R_F values of malic acid gave results from 0.53 to 0.57, the average being 0.55.

RESULTS AND DISCUSSION

Formate Columns

Emergence of Known Acids

The elution pattern for a series of pure organic acids is shown in Figure 3 which is a composite of several separate experiments each with 2 to 5 components present. The data concerning the dimensions of the column, eluting agent, etc., are given in the legend. The peak volume values (defined as volume eluted at the peak titration value of the particular acid) for a series of known acids are summarized in Table 2. These values are generally reproducible within 3 to 6 ml., but occasional deviations of the order of 10 ml. have been noted.

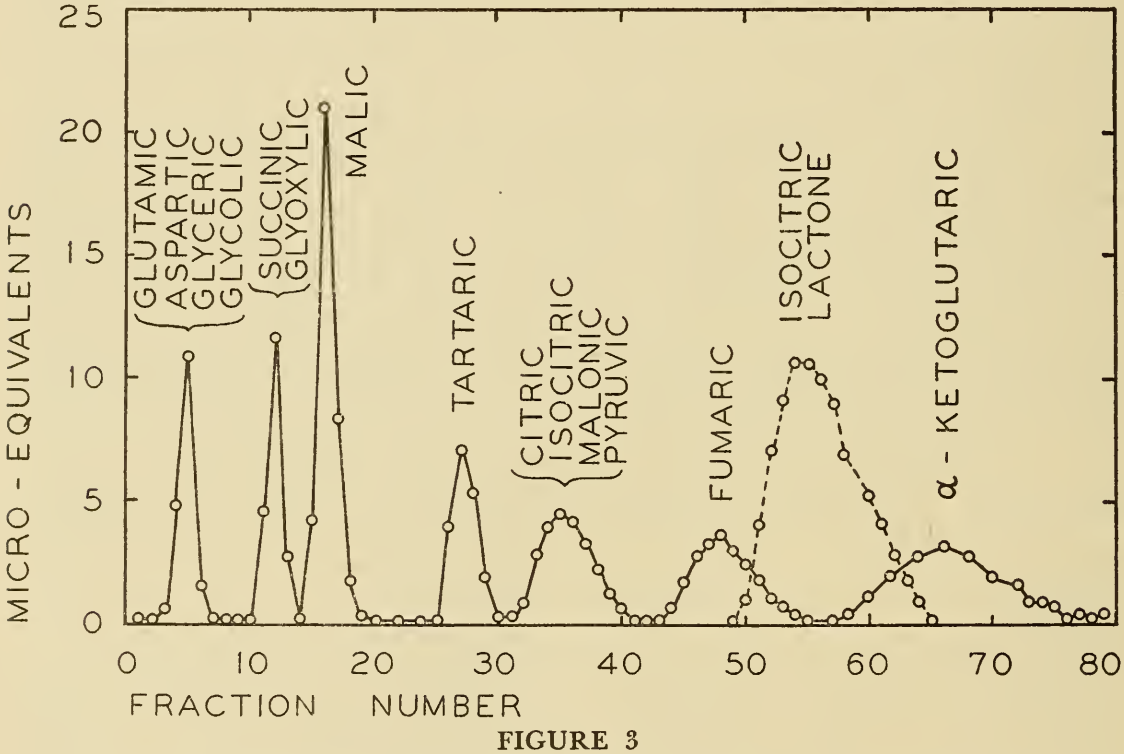

FIGURE 3

Composite diagram of the ion exchange separation of pure organic acids. Dowex 1-X10, 200 - 400 mesh, formate form, 0.28 sq. cm. x 5.5 cm. Gradient elution, 3.5 *N* formic acid in reservoir; flow rate, 1 ml. per minute; fraction volume, 2.5 ml.

[1]The indicator solution is made by dissolving the bromophenol blue in 95 per cent ethyl alcohol and adding 1 *N* sodium hydroxide drop by drop with stirring until the color is reddish blue by transmitted light. The spraying device (Research Equipment Corporation, Oakland, California, catalogue No. MJ50) is operated with compressed air.

TABLE 2. APPROXIMATE PEAK VOLUMES OF ORGANIC ACIDS ELUTED FROM ION EXCHANGE COLUMNS

Dowex 1-X10, 200 - 400 mesh. Flow rate 1 ml. per minute. Gradient elution. Normality of acid in reservoir and dimensions of columns as noted.

Acid	Peak volumes			
	Formate columns		Acetate columns	
	5.5 cm. x 0.28 sq. cm. 3.5 *N* Formic acid	4.0 cm. x 0.28 sq. cm. 3.5 *N* Formic acid	6.0 cm. x 0.28 sq. cm. 2.5 *N* Acetic acid	11.5 cm. x 0.78 sq. cm. 6.0 *N* Acetic acid
	ml.	ml.	ml.	ml.
Glutamic	10		18	36
Aspartic			28	48
Glyceric	13	11	56	94
Glycolic				
Glyoxylic	30		102	
Succinic				138
Malic	40		118	
Tartaric	67			
Citric	88	70		
Pyruvic				
Isocitric				
Malonic				
Fumaric	120	93		
Isocitric lactone	140			
α-ketoglutaric	165	120		
Maleic[1]		130		

[1]8 *N* formic acid in reservoir.

The plot of the titration of the acids that are eluted early in the procedure exhibits sharp peaks which approach the theoretical symmetrical form but, as the elution progresses, the peaks become broader.[1] Accordingly, it is more difficult to separate the acids that occur later on the elution diagram. Nevertheless, if these acids are of interest, it is only necessary to increase the concentration of the formic acid in the reservoir, either initially or at some time during the course of the elution. The effect is to elute the acids sooner and also to sharpen the peaks. For example, with 6 *N* formic acid in the reservoir and other conditions the same as in Figure 3, the peaks for malic, citric, fumaric,

[1]Lakshmanan and Lieberman (11) have recently described a gradient elution technique in which the rates of flow into and out of the mixing chamber are independently variable. They showed that the later peaks can often be sharpened considerably by adjustment of the two flow rates. The adjustment is made so that the rate of flow of stronger acid into the mixing chamber is less than the rate at which diluted acid is flowing into the column. A plot of the concentration of the acid flowing into the column against the total volume delivered thus yields a curve that is concave upwards.

and α-ketoglutaric acids occur in the position shown on Figure 4. The malic acid peak has been shifted from fraction 16 to 10, the citric acid peak from fraction 35 to 21, and the α-ketoglutaric acid peak from fraction 66 to 37. Furthermore, the peaks are more symmetrical than those in Figure 3.

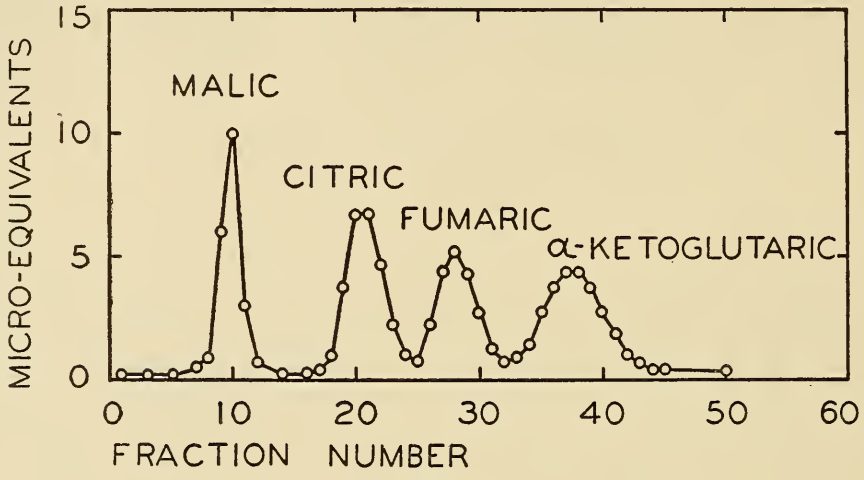

FIGURE 4

Ion exchange separation of pure organic acids. All conditions as in Figure 3 except that 6 *N* formic acid was used in the reservoir.

The acids which are eluted in advance of malic acid may overlap when using higher concentrations of formic acid but, by proper adjustment of the column length and the concentration of the formic acid in the reservoir or both, or by use of the acetate system described later, it is possible to separate most mixtures of acids. However, citric, isocitric, malonic, and pyruvic acids have not yet been successfully separated from each other by the technique as at present developed.

The recoveries of individual acids from the formate ion exchange columns are summarized in Table 3 and are, with a few exceptions, satisfactory. Glyceric acid gave low recoveries after titration of the dried effluent, and the pooled effluent did not give satisfactorily quantitative results by any colorimetric procedure tried. The consistently high results given by malonic acid have not yet been accounted for. With α-ketoglutaric acid, both titration and colorimetric procedures gave low results.

Busch, Hurlbert, and Potter (4) have reported that only two titratable acid groups per molecule were available for titration after the emergence of citric acid from a column of anion exchange resin 0.78 sq. cm. in cross section and 11.5 cm. in height. On the other hand, essentially complete recoveries were obtained if the fractions were analyzed colorimetrically. In the present study, the yields of citric acid, based on titration of the residue after evaporation of the formic acid, averaged 98 to 100 per cent when eluted from columns 0.28 sq. cm. in cross section and 3 to 6 cm. in height. However, recoveries of titratable citric acid have been found to vary between the limits of 60 to 90 per cent when longer columns were used. No explanation of this phenomenon will be attempted, but it is apparent that the yields are dependent on

TABLE 3. RECOVERY OF KNOWN ACIDS FROM FORMATE ION EXCHANGE COLUMNS

Acid	Number of experiments	Average recovery	Range
		per cent	per cent
Glutamic	2	100	100
Aspartic	2	102	100-104
Glyceric	6	83	80-92
Glycolic[1]	2	98	94-102
Succinic	3	100	100-101
Glyoxylic[1]	1	102	
Malic	10	99	97-101
Tartaric	5	99	97-102
Citric[2]	5	101	98-102
Malonic	2	123	121-125
Pyruvic[1]	1	104	
Fumaric	5	99	90-105
α-ketoglutaric	4	80	74-88
Maleic	2	100	98-102

[1]Recoveries obtained when fractions containing the acid were pooled and determinations made by specific techniques. All other recovery values were obtained by titration after evaporation of the effluent.

[2]Recoveries vary with column dimensions. See text.

column height and caution must be exercised when determining citric acid by the present technique.

Determination of the Organic Acids of Tobacco Leaf

General. For routine determination of the organic acids of tobacco leaf tissue, a column 4.0 cm. x 0.28 sq. cm. provides a rapid separation of the acids of major interest when gradient elution is carried out with 3.5 *N* formic acid in the reservoir. Figure 5 is a typical elution diagram obtained from dried green tobacco leaf under these conditions. The final peak has been labeled "citric peak" for purposes of discussion; however, it represents not only citric acid but also an acid of as yet unknown identity. Both water and ether extracts yield essentially the same elution pattern when 2 to 4 ml. (*ca.* 0.2 m.eq.) of the extract are added to the column. The resin is discolored by the extract, and green or yellow material occasionally passes down through the column during the addition of the sample and the washing operation. However, this does not affect the subsequent separation of the acids.

After elution, all of the fractions are evaporated to dryness and titrated. The time required, counting from the addition of the sample to the column to the titration of the 30th fraction, is approximately 3 hours. Additional time is needed for the citric acid analysis on the pooled "citric peak" fractions, but this may be reduced to a minimum by collecting the fractions from a series of samples and analyzing them as a group at a later date. The identity of the unknown acid that occurs together with citric acid on the elution pattern is presently under study.

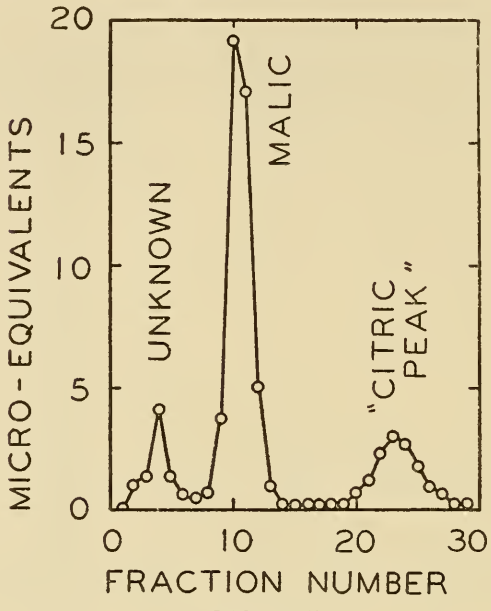

FIGURE 5

Ion exchange separation of the nonvolatile organic acids of green tobacco leaf. Dowex 1-X10, 200 - 400 mesh, formate form, 0.28 sq. cm. x 4.0 cm. Gradient elution, 3.5 *N* formic acid in reservoir; flow rate, 1 ml. per minute; fraction volume, 3.0 ml. Sample - 2 ml. of water extract of dried tobacco leaf.

For many practical applications, the presence of 10 to 20 per cent of unknown acidity in the "citric peak" would not invalidate the conclusions drawn from the data, and an approximate value for citric acid could be estimated by the direct titration technique. However, in the case of tobacco, the quantity of the unknown acid may amount to as much as 40 per cent of the acidity of the pooled "citric peak" fractions and, accordingly, the citric acid must be determined separately. There are several rapid colorimetric procedures which doubtless could be used for the determination of citric acid in this case since the analysis is carried out on solutions which contain only a single acid impurity. However, for the present investigation the more precise titrimetric procedure of Hargreaves, Abrahams, and Vickery (9) has been preferred.

The analysis of the effluent from a single column gives data for malic, citric, and undetermined acids. The sum of these plus the separately determined oxalic acid yields a value for the total nonvolatile organic acids since no significant quantity of acid is eluted beyond the acids in the "citric peak". It should be emphasized that the acids determined by the present procedure comprise only the major acid components of the tobacco leaf tissue. Volatile acids and such unstable substances as α-keto acids are not included. However, these substances are present in only minor proportions.

The system has been satisfactory for the analysis of ordinary green tobacco leaf samples, as the elution pattern of a wide variety of samples has differed only slightly from that of Figure 5. On the other hand, when excised leaves which had been cultured in organic acids other than malic or citric acid have been analyzed, it has been necessary to modify the procedure. For example, in a recent experiment in which tobacco leaves were cultured in (+)-tartrate (20), the column length had to be in-

creased to 5.5 cm. (see Figure 3) in order to achieve the optimum separation of this substance from malic and citric acids. It was also necessary to lengthen the column for the analysis of leaves that had been cultured in succinate (20) and accordingly contained 5 to 20 times the normal amount of this substance. The ready adaptability of the technique to variation in the identity and quantity of the acids in the samples is one of the most desirable features of this general method. A few trial runs on any new set of samples usually provides sufficient information to permit the analyst to set up the series of determinations.

Tobacco leaves that have been cured and fermented as in commercial practice differ appreciably in their organic acid composition from the freshly picked leaf (19). Figure 6 shows the elution pattern obtained with a water extract of a sample of dried tobacco leaf tissue that had been cured and fermented under the conditions employed in Connecticut for the preparation of cigar wrappers. The sample analyzed was the one labeled FJ in a previous bulletin from this laboratory (19) where the treatment of the sample is thoroughly described.

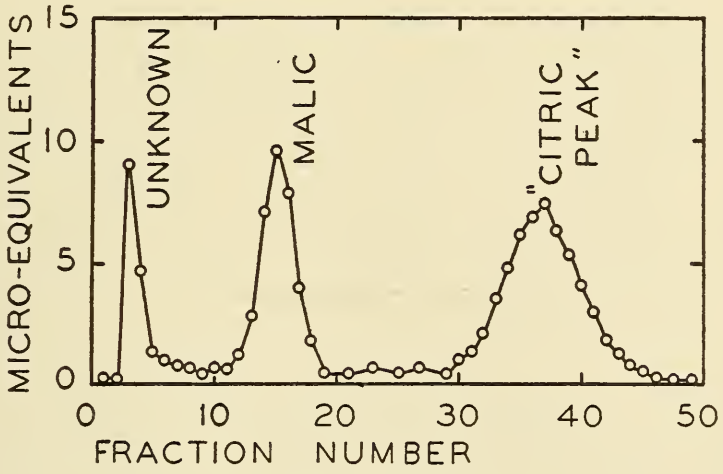

FIGURE 6

Elution diagram of the nonvolatile organic acids of cured and fermented tobacco leaf. Dowex 1-X10, 200 - 400 mesh, formate form, 0.28 sq. cm. x 5.5 cm. Gradient elution, 3.5 *N* formic acid in reservoir; flow rate, 1 ml. per minute; fraction volume, 2.5 ml. Sample - 2 ml. of water extract of dried tobacco leaf.

The elution pattern of Figure 6 indicates that the nonvolatile organic acids of the cured and fermented tobacco may be determined as readily as in the dried green leaf. The major quantitative change in the organic acids that occurs during curing involves a transformation of approximately one-half of the malic acid originally present to citric acid.

An additional feature of the elution pattern of the cured and fermented leaf is the considerable increase in the "unknown" acidity over that observed in the green leaf. Evidence has been obtaind from ninhydrin color tests and paper chromatography that this increase arises mainly from the presence of aspartic acid. During the course of fermentation, the asparagine amide nitrogen in this series of samples decreased by about 33 m.eq. per 100 gm. dry tissue (19), and the appearance of an equivalent quantity of aspartic acid would therefore be anticipated.

The peak labeled "unknown" in Figure 6 contains acidity equal to about 41 m.eq. Another aliquot of the original extract was accordingly analyzed on an acetate column as described in a later section. Acidity corresponding to 27 m.eq. was found in the position occupied by aspartic acid in control experiments, and the fractions gave positive ninhydrin reactions of intensity roughly proportional to the titrations. The identity of the component as aspartic acid therefore seems highly probable. The other small peaks on the elution curve from the acetate column which represent the acids that emerge in advance of malic acid from the formate column had a combined acidity of approximately 17 m.eq. The marked increase in the size of the "unknown" peak in Figure 6 over the analogous peak observed when analyzing green tobacco leaves (see Figure 5) is thus accounted for.

It should be pointed out, however, that if ether extracts of cured and fermented tobacco were examined, no increase in the "unknown" peak would be anticipated since aspartic acid is not extracted from acidified tobacco tissue with ether.

Precision and Accuracy of the Analytical Technique. The precision of the analytical technique may be judged from the results given in Table 4 which shows data obtained in repeated analyses of a single uniform sample of green tobacco leaf tissue. In all cases, the error in the determinations (as measured by the standard deviation and coefficient of variation) arises from two main sources. The first is the extraction procedure and the second the analytical operation which is carried out on an aliquot of the extract. Analysis of variance was used to estimate the contribution of each of these sources of error to the standard deviation term.

From Table 4 it is apparent that the precision of the malic acid determinations by the ion exchange technique on either water or ether extracts shows considerable improvement over the chemical method (15, 16) which has been used in this laboratory. The analysis of variance of the data obtained by the chemical procedure showed that the standard deviation for the determination itself, exclusive of the error contributed by the extraction method, was ±3.90 m.eq. per 100 gm. dry weight. This may be compared with a standard deviation of ±0.93 m.eq. per 100 gm. dry weight for the data obtained by the ion exchange technique on water extracts.

It could also be concluded that the part of the total error contributed by the extraction procedure was statistically insignificant in relationship to the analytical error of the chemical method. However, this source of error becomes significant in relationship to the smaller standard deviation of the data obtained by the ion exchange technique. Improvements in extraction procedure could possibly be made if the tissue were more finely ground, but this involves additional technical difficulties.

The results for citric acid in Table 4 show a considerably higher precision in the analysis of water extracts than of ether extracts. Since the same titrimetric procedure (9) is used for the final analysis whether ion exchange separation is carried out or not, it is surprising to find that the standard deviations are ±1.0 m.eq. per 100 gm. dry tissue for

TABLE 4. PRECISION OF THE DETERMINATIONS OF ORGANIC ACIDS IN A REPRESENTATIVE SAMPLE OF DRIED GREEN TOBACCO LEAF TISSUE

	Determination	Number of replicates[1]	Mean±standard deviation m.eq. per 100 gm. dry wt.	Coefficient of variation per cent
Ion exchange method water extracts	Malic acid	10	120.1±1.69	1.4
	"Citric peak" acidity	10	34.5±1.5	4.3
	Citric acid[2]	10	20.4±0.5	2.4
	Total organic acids	10	208.1±2.8	1.3
	Undetermined acids	10	33.6±2.4	7.1
Ion exchange method ether extracts	Malic acid	12	122.7±1.66	1.4
	"Citric peak" acidity	10	32.4±2.5	7.7
	Citric acid[2]	9	20.9±1.0	4.8
	Total organic acids	10	208.6±6.1	2.9
	Undetermined acids	11	31.5±5.1	16.2
Chemical methods (9, 15, 16) ether extracts	Malic acid	8	120.1±6.56	5.5
	Citric acid[3]	15	22.5±1.0	4.4

[1]Analysis in duplicate of at least 5 separate extractions of the tissue in all but two cases. The coefficients of variation thus include not only the errors of the ion exchange technique but also those of the extraction procedure.

[2]The values for citric acid represent determinations by the method of Hargreaves, Abrahams and Vickery (9) on the pooled "citric peak" fractions from the columns.

[3]A sufficient number of citric acid analyses of this tobacco sample was not available for statistical analysis. Triplicate determinations of citric acid on a series of 5 samples of Bryophyllum leaf were accordingly used to calculate the value in the table. The analytical data for total solids, ash, total nitrogen, protein nitrogen, and starch on this group of samples had been subjected to statistical analysis (18) and the uniformity of the composition thoroughly established.

the analysis of ether extracts and only ±0.5 m.eq. per 100 gm. for the analysis of water extracts. The analysis of variance of the citric acid determinations furnished the explanation of this since it was found that the ether extraction of citric acid from tobacco leaf tissue introduces a significant error into the determination. The standard deviation of the titrimetric method itself, exclusive of the error due to the extraction procedure, is approximately ±0.7 m.eq. per 100 gm. dry weight.

The total organic acid determinations show a low coefficient of variation for both types of extracts, although the results with ether extracts are somewhat less precise. The figures in Table 4 were obtained by addition of the acidity of all of the fractions after gradient elution. Similar results are obtained with the rapid procedure for total organic acids described on page 10.

The coefficients of variation of the data for both the undetermined acids and for the "citric peak" acidity are considerably higher than those for the other determinations. This is to be anticipated since the "citric

peak" contains at least one other substance of unknown properties, and the identity of only a few of the minor components represented by the data for undetermined acids is known. This is not a serious defect of the general method, and the errors can probably be minimized when the unknown acids have been identified. Furthermore, the sum of the unknown acids is certainly more precisely estimated by the ion exchange procedure than by the chemical methods hitherto used because of the improved accuracy of the determination of total acidity.

In order to estimate the accuracy of the chromatographic method, the results obtained for malic and citric acid by this method were compared to those obtained by the previously used chemical methods (9, 15, 16). The mean of the malic acid determinations was 120.1 m.eq. per 100 gm. of dry weight (10 determinations) by the chromatographic method on the water extract and 122.7 m.eq. per 100 gm. as determined on ether extracts. The mean of 8 determinations by the previously used method was 120.1 m.eq. per 100 gm.

The mean of the data for citric acid was 20.4 m.eq. per 100 gm. of dry weight (10 determinations) as determined by the chemical method on pooled chromatographic fractions when water extracts of the tissue were analyzed and 20.9 m.eq. per 100 gm. (9 determinations) on ether extracts. On the other hand, the result of direct chemical analysis of ether extracts was 22.6 m.eq. per 100 gm. (6 determinations) and of water extracts 22.1 m.eq. per 100 gm. (5 determinations). It may be inferred that water is as effective in extracting citric acid from the tissue as ether but there is a small but significant discrepancy between the results of the chromatographic and the direct method.

The yields, when known quantities of citric acid are placed on the columns, eluted, and the fractions carried through the complete analytical procedure, have been 98 to 102 per cent. Since recoveries of citric acid are dependent on column characteristics and dimensions (see page 16), the possibility exists that unknown components of the extract may so change the properties of the resin as to cause losses of citric acid.

In order to test this hypothesis, citric acid analyses by the ion exchange technique (including chemical determination on the pooled fractions) were carried out on samples of tobacco extract to which known amounts of citric acid had been added. The recoveries of the added citric acid were in the range of 97 to 99 per cent which indicates that losses of the order of 1 to 3 per cent may occur. However, the differences involved when comparing the results of the two methods are somewhat greater than 3 per cent, and it seems probable that the direct chemical method when applied to a tobacco extract yields a slightly high value for the citric acid content. A possible explanation is the presence of a compound (or compounds) in the tobacco extract which, after oxidation and bromination, is carried through the procedure and leads to an increase in the final iodine titration. With either method, the error involved is not great and would affect few conclusions which could be drawn from the results obtained on plant tissue.

The method for total organic acids described in an earlier paper (16) has served for many years to indicate the trends in the total acidity

of leaf samples subjected to various treatments. However, the precision of the technique has never been entirely satisfactory, owing to the difficulty of obtaining sharp end points when titrating a complex mixture of organic acids between arbitrarily and empirically chosen pH limits in the presence of small quantities of sulfuric acid. It has become increasingly apparent that the results cannot be considered to be an accurate measurement of the true organic acidity of the plant tissue. Thus, it is meaningless to compare the results obtained by the ion exchange technique with those by the previous method. The data in Table 4 clearly show that the ion exchange technique provides highly reproducible values for the total organic acidity arising from stable, nonvolatile acidic components. However, further study will be necessary to determine to what degree the results obtained by the chromatographic technique represent the actual total organic acidity of the tobacco leaf.

Effect of Resin Properties on the Separation of Malic and Citric Acids

Table 5 contains a summary of the peak fraction and limit fractions (i.e., the fraction numbers between which the substance is eluted) of the malic and citric acid peaks obtained under various conditions of crosslinkage and mesh size of the resin. The sample used was a water extract of dried tobacco leaf. These data were obtained while studying the optimum conditions for separating the acids, and are presented as an illustration of the effects of these variables and as an aid in future application of the ion exchange technique.

TABLE 5. EFFECT OF CROSSLINKAGE AND MESH SIZE OF ION EXCHANGE RESIN ON ELUTION BEHAVIOR OF MALIC AND CITRIC ACIDS

Dowex 1, formate form, 4.0 cm. x 0.28 sq. cm. Flow rate 1 ml. per minute. Fraction volume 3 ml. Gradient elution, 3.5 *N* formic acid in reservoir. Sample - water extract of green tobacco leaf tissue.

Nominal mesh size	Degree of crosslinkage	Malic acid		Citric acid	
		Peak fraction	Limiting fractions	Peak fraction	Limiting fractions
200 - 400	X 10	13	10 - 16	30	26 - 36
200 - 400	X 4	9	7 - 12	17	14 - 21
200 - 400	X 2	7	5 - 10	14	11 - 20
100 - 200	X 10	10	7 - 15	24	18 - 30

The degree of *crosslinkage* has an appreciable effect on the separation and sharpness of the acid peaks. Moore and Stein (12) have found Dowex 50-X4 to yield optimal separations of amino acids, and the results obtained in the present work with Dowex 1-X4 indicate that this degree of crosslinkage is advantageous for certain specific separations of organic acids. Further testing will be necessary before definite conclusions can be drawn.

Mesh size[1] also influences the separation. The malic and citric acid peaks move off the 100-200 mesh resin several fractions earlier than from the 200-400 mesh resin and the peaks are noticeably broader.

[1]The figures given are the nominal mesh sizes as supplied by the manufacturer. The resins used in these studies had been modified by removal of the finer particles by decantation.

The *flow rate,* up to about 2 ml. per minute, does not appreciably affect the separations. Flow rates higher than this have not been tried since excessive pressures (5 pounds per square inch or higher) are necessary to produce them.

Variations in the separation of the acids occur from batch to batch of resin. These are probably due to distribution of mesh size and variations in crosslinkage in the different batches of resin. The actual mesh size range is particularly hard to maintain constant. It is necessary to decant the "fines" in order to provide a sufficiently rapid flow rate, and the proportion of resin decanted on different occasions may vary. Another variable to be taken into consideration is the time of storage of the resin following its conversion to the desired formate or acetate form. Storage periods of several months apparently bring about some deterioration in efficiency.

However, entirely satisfactory separations and analyses of the most important nonvolatile organic acids of tobacco leaf are obtained within rather wide limits of these variables. Where additional acids are involved it may be necessary to control such variables as these within narrower limits.

Acetate Columns

Emergence of Known Acids

On the formate columns, two groups of acids that are eluted in advance of malic acid were not satisfactorily separated (see Figure 3). Busch (3) has described a method for improving the separation of several of the faster moving acids with use of a column of resin in the acetate form and acetic acid as eluting reagent. This technique has accordingly been applied to the group of acids that are eluted from the formate column in advance of malic acid.

Figure 7 shows the elution pattern of a known mixture of glutamic, aspartic, glyceric, glycolic, and succinic acids from a 6.0 cm. x 0.28 sq. cm. resin column. Malic acid emerges approximately between fractions 55 and 67 under these conditions. The separation of several of the faster moving acids is clearly possible with this system, and the elution may be carried out in a minimum of time. Resin columns of this size may be satisfactory for many purposes. However, for tobacco leaf extracts, the fast moving acids comprise only about 10 per cent of the total acidity, and the small columns become overloaded[1] with other anions present in the extracts (malate, citrate, $SO_4^=$, NO_3^-, etc.) before enough of the minor acids to provide for accurate titration values can be added.

In order to obtain the required capacity, larger columns have accordingly been used. Figure 8 shows the separation of a group of the faster moving acids which is obtained on a 11.5 cm. x 0.78 sq. cm. (1 cm.

[1]Overloaded in the sense that a breakdown in the separating ability of the column becomes apparent even though the total capacity of the resin (1.2 m.eq./ml.) has not been exceeded. The separations seem to be better when the column loading is held below 25 per cent of total capacity.

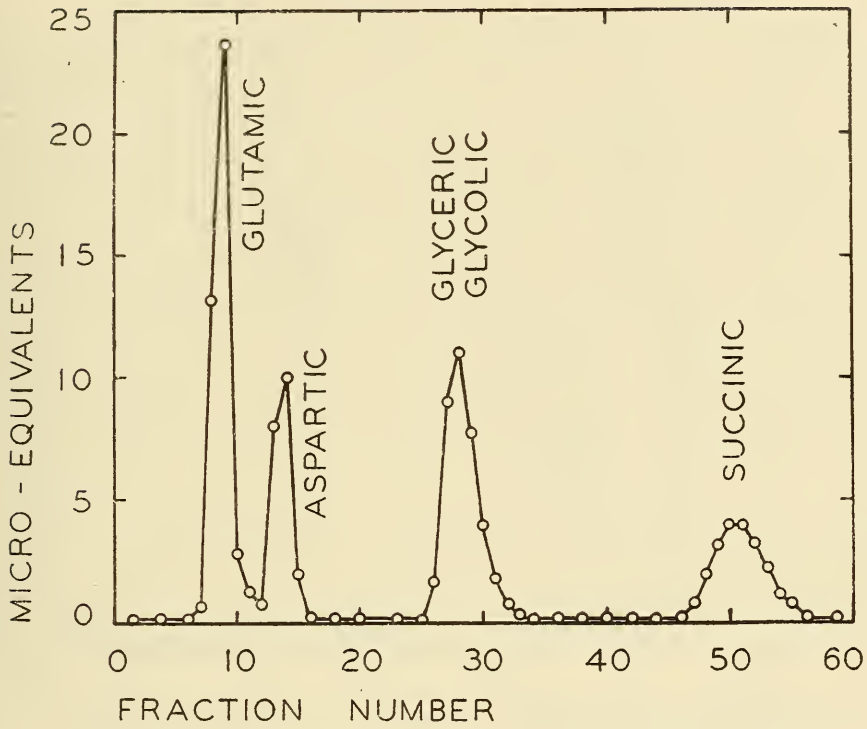

FIGURE 7

Ion exchange separation of pure organic acids. Dowex 1-X10, 200 - 400 mesh, acetate form, 0.28 sq. cm. x 6.0 cm. Gradient elution, 2.5 *N* acetic acid in reservoir; flow rate 1 ml. per minute; fraction volume, 2.0 ml.

diameter) column; the elution pattern is essentially unchanged and choice between a small or large column thus depends entirely on the composition of the sample to be analyzed.

The Fast Moving Acid Components of Extracts of Green Tobacco Leaf

Paper chromatographic analysis of the fractions comprising the unknown acid peak that occurs before malic acid in the formate system when analyzing tobacco extracts revealed the presence of several acids. Figure 9 shows the result of eluting a sample of tobacco leaf extract from an acetate column in an attempt to separate these acids. The separation is far from complete, but several peaks appear and, for the purpose of discussion, they have been numbered in order on the elution diagram. Paper chromatograms have revealed that peaks 4 and 5 each represent two components. Peak 6 has been tentatively identified as succinic acid. The other peaks have not yet been investigated, but it is apparent that the fast moving unknown acids of the tobacco leaf extract comprise a mixture of at least seven distinct substances. Attempts to separate the acids of peaks 4 and 5 by adjustment of the elution conditions have so far met with little success. Work is continuing on the identification of these acids. As their identities become known, it will become possible to devise specific methods to determine them and to study their role in the metabolic system of the leaf.

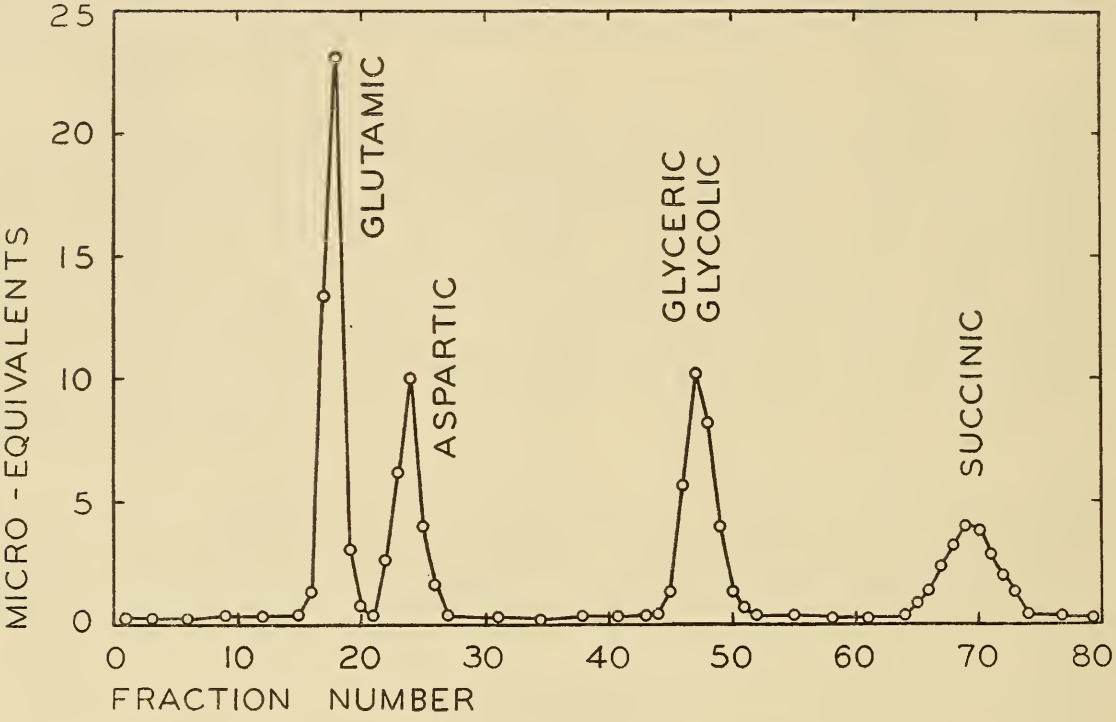

FIGURE 8

Ion exchange separation of pure organic acids. Dowex 1-X10, 200 - 400 mesh, acetate form, 0.78 sq. cm. x 11.5 cm. Gradient elution, 6.0 *N* acetic acid in reservoir; flow rate, 1 ml. per minute; fraction volume, 2.0 ml.

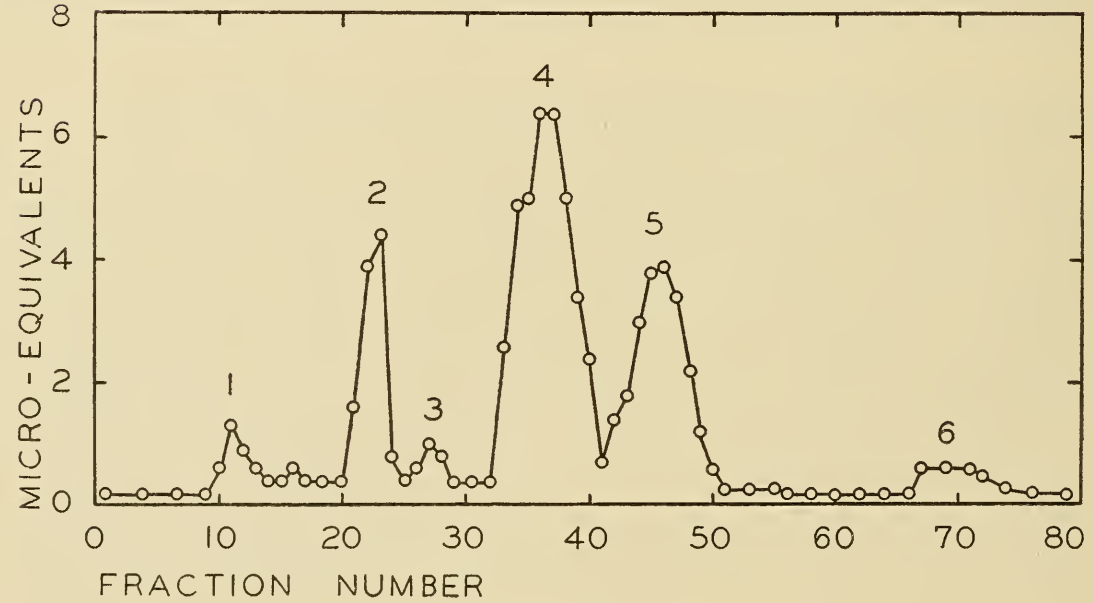

FIGURE 9

Ion exchange separation of fast moving, nonvolatile organic acid components of green tobacco leaf. All conditions as in Figure 8. Sample - 10 ml. of water extract of dried tobacco leaf.

Identification of Acids Emerging from Column

The position of known acids on the elution diagram was first established by applying known mixtures of acids to the column and by reference to the published results of Busch, Hurlbert, and Potter (4). Identification of the several peaks of acidity that are observed with tobacco leaf extracts was tentatively made by comparing the position of the peaks with those of known acids. These identifications were corroborated by specific color tests (7), by analysis of the appropriate pooled fractions for malic and citric acid by an independent chemical method (9, 15, 16), as well as by paper chromatography side by side with authentic samples of the suspected acids. Identification in these terms was considered sufficient for those acids that are known to occur in tobacco tissue in relatively large quantities or for those acids that were introduced into the leaf by the excised leaf technique (20).

Tentative identifications of several of the unknown acids have been made with use of these criteria, but positive identification awaits isolation in pure form in sufficient quantities for determination of physical properties and preparation of derivatives.

Application of Ion Exchange Chromatography to the Separation of Organic Acids of Other Plant Tissues

The ion exchange separation of organic acids would appear to have general applicability to the analysis of organic acids in plant tissues and of commercial products derived from them wherever a rapid, precise, and accurate method is desired. For example, the method can undoubtedly be employed with use of the conditions described in the legend of Figure 3 for the rapid determination of malic, tartaric, and citric acids in products derived from grapes. This particular determination has heretofore been difficult and time-consuming.

As a preliminary trial of the general applicability of the technique, the organic acids of several samples of plant tissues and commercial products have been separated on the formate columns. A diagram of the elution patterns of four of these is presented in Figure 10. Estimates of the accuracy and precision of analyses in tissues other than tobacco have not as yet been attempted. The identification of the acids depends upon R_F values on paper chromatograms and on the relative position of the peaks on the elution diagram. The elution patterns serve to illustrate the similarities and differences in the organic acid content of the several materials.

Comparison of Ion Exchange Chromatography with Partition Chromatography on Silica Gel

Ion exchange chromatography possesses a number of advantages over partition techniques for the separation of the acids of biological interest. First, the acids may be added to the resin column in aqueous solution, and the volume of the sample added is limited only by practical considerations as long as the effective capacity of the resin is not exceeded. Cationic components and such substances as amino acids and sugars

can then be washed away with water. On the other hand, when working with silica gel columns, it is essential to carry out a preliminary removal of cations and then dissolve the sample in a small volume of an organic solvent such as chloroform, in order to minimize undesirable effects upon the equilibrium between free water and the water bound by the particles of gel when the sample is added to the column. The choice of organic solvents is limited because of solubility considerations. Addition of aqueous samples directly to a silica gel column is possible (21), but the volume must be kept below 0.5 ml. and the addition must be made under carefully controlled conditions. Thus, the preparation of extracts of dry or fresh plant tissue suitable for analysis on the silica gel column is difficult and time-consuming, particularly if quantitative results are desired.

Second, the anion exchange resins possess many times the effective capacity of silica gel. Accordingly, analysis for components which com-

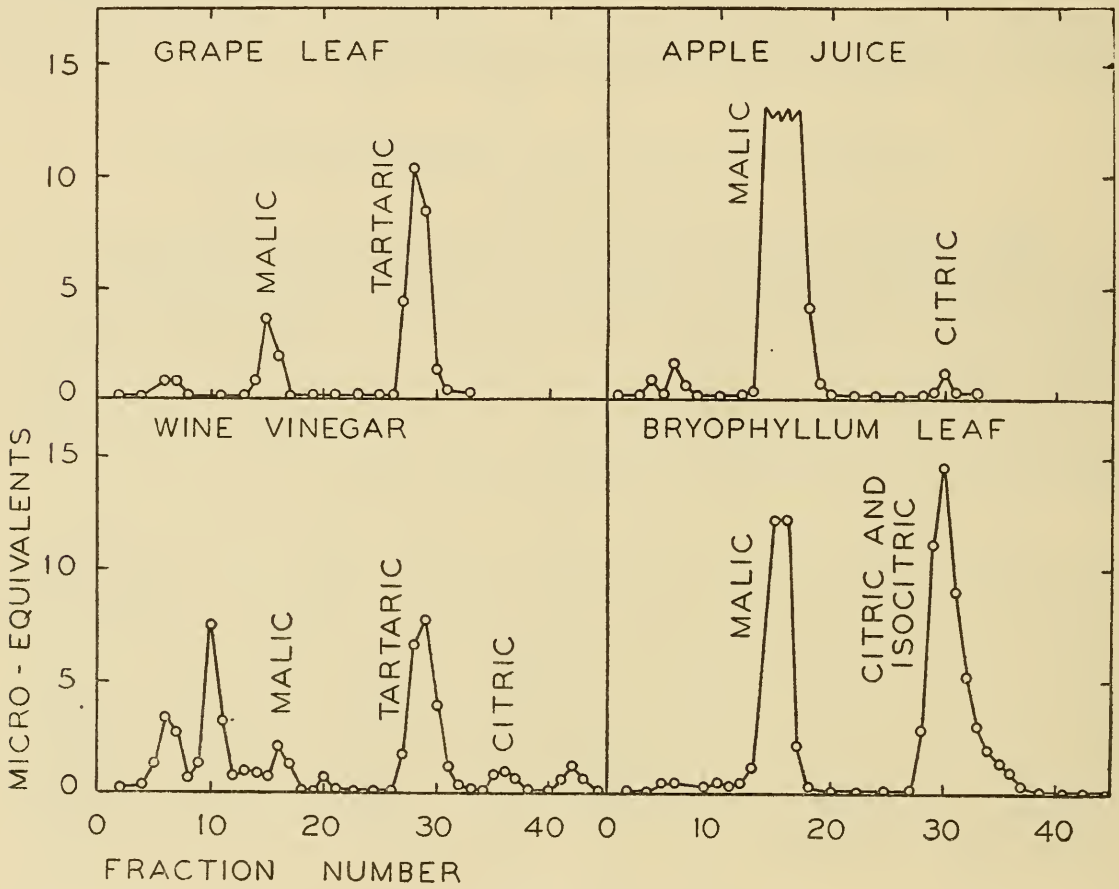

FIGURE 10

Ion exchange separation of the nonvolatile organic acids of some plant tissues and commercial products derived from plants. Dowex 1-X10, 200 - 400 mesh, formate form, 0.28 sq. cm. x 6.0 cm. Gradient elution, 3.5 *N* formic acid in reservoir; flow rate, 1 ml. per minute; fraction volume, 2.5 ml. Samples - The leaf materials were dried and extracted with ether as described for tobacco. Apple juice was used as received. The wine vinegar was evaporated to dryness to remove acetic acid; the residue was diluted with water to the original volume before adding an aliquot to the column.

prise only a small proportion of the total acidity becomes possible. A high capacity is also valuable where the isolation of an acid for identification purposes is desired.

Third, the titrations of the effluent from the ion exchange columns are carried out in aqueous solutions of the free acids rather than in a two-phase system that contains measurable amounts of sulfuric acid. Sharp end points and low blank titrations are thus secured. Finally, the acid peaks on the elution diagrams from the ion exchange columns are, in general, narrower and more symmetrical than those from the partition columns. The shape of the peaks obtained from a silica gel column is highly dependent on maintenance of the water content both of the gel and of the eluting solvent within narrow limits. Thus, temperature fluctuations during the course of elution from a silica gel column may markedly alter the separations.

Perhaps the major disadvantage of the ion exchange technique for analytical purposes is the fact that fractions which contain volatile or unstable substances (e.g., acetic acid, pyruvic acid) cannot be merely evaporated to dryness and titrated, but must be pooled and the acids determined individually by some other procedure.

For many purposes, the ion exchange and silica gel techniques supplement each other, as organic acids that are inseparable by the one method can often be satisfactorily separated by the other. Where nonvolatile and relatively stable acids are chiefly involved, the ion exchange technique has definite advantages, especially for routine analysis, and, because of its versatility and flexibility, is also to be preferred for the survey of plant materials of unknown organic acid composition. For isolation purposes, the high capacity of the resins makes them far superior to silica gel.

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SUMMARY

A technique is presented for the rapid, precise, and accurate determination of malic and citric acids and of the total nonvolatile organic acidity in water or ether extracts of dried tobacco leaf tissue. The acids are separated by chromatography on anion exchange resins (Dowex 1-X10, formate) by the use of gradient elution with continuously increasing concentrations of formic acid. The quantity of organic acid in each of the

fractions of effluent is determined by titration of the residue that remains after evaporation of the eluting agent in a stream of air at 46 - 48° C.

A plot of the individual titrations shows a succession of peaks of which one represents exclusively malic acid. A second peak represents citric acid together with another as yet unidentified acidic component. These fractions are therefore pooled and citric acid is determined in the mixture by the technique of Hargreaves, Abrahams, and Vickery (9). The sum of the acidity of all of the effluent fractions, together with the separately determined oxalic acid, represents the "total organic acids", and the "undetermined acids" are taken as the difference between this sum and the sum of the malic, citric, and oxalic acids.

A statistical analysis of the results obtained with this technique and also of data obtained by the chemical methods previously used in this laboratory (9, 15, 16) showed the ion exchange technique to be superior for the determination of malic acid and of the total acidity. The results for citric acid are of about equal precision and accuracy by both methods.

The separation and determination of many other organic acids of interest in metabolism is also possible with the formate system, and modifications can readily be introduced to make the method suitable for application to the analysis of any plant tissue which can be suitably prepared. As evidence of this possibility, authentic samples of a wide variety of acids have been tested and recoveries in the range of 97 to 102 per cent have been obtained.

Anion exchange chromatography on Dowex 1 in the acetate form with continuously increasing concentrations of acetic acid as the eluting agent makes possible the separation of several fast moving organic acids which are eluted as mixtures from the formate column. Preliminary analysis on the acetate resin of the fast moving organic acids of the tobacco leaf provides evidence that there are seven or more components in the "undetermined acids" fraction.

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